

The University of Chicago

STUDIES ON THE ENZYMATIC
FORMATION AND DESTRUCTION
OF URIC ACID IN MAMMALIAN
BLOOD

AN EXPANSION OF A DISSERTATION SUBMIT-
TED TO THE FACULTY OF THE DIVISION OF
THE BIOLOGICAL SCIENCES IN CANDIDACY FOR
THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF BIOCHEMISTRY

1939

By
MARY BRANNOCK BLAUCH

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A NEW METHOD FOR THE DETERMINATION OF URIC ACID IN BLOOD, WITH URICASE*

By MARY BRANNOCK BLAUCH

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The original Folin and Denis (2) phosphotungstate colorimetric method for uric acid was increased in sensitivity through the accidental discovery by Benedict (3) and Hitchcock (3) that cyanide ion materialized a color developed in the reaction. The color was increased by heating (4). The net result of the modification required was decreased from 2 cc. to 1 cc. of reagent. Benedict nor Folin appears to have applied the ratio of uric acid to uric acid reagent. The sensitivity of the reaction (5).

Many attempts have been made to improve the specificity of the color reaction. Improvements in the preparation of the uric acid reagent devised by Folin (6) and Benedict (4) did not meet the high degree of specificity actually required. Neither did the attempts at preliminary precipitation of uric acid as silver urate (7) nor the attempts to eliminate glutathione and ergothioneine by preparing unlaked blood filtrates (8) prove entirely satisfactory. To date the method of Newton (9), in which the glutathione and ergothioneine are removed by the Benedict procedure (7), urea is added to the cyanide solution to prevent turbidity (8), and a lithium arsenotungstate is used as the reagent, appears to be the best modification of the colorimetric method for uric acid. Nevertheless, an improved specificity of the reaction, a better per cent of recovery of uric acid, and simplification of procedure seemed desirable.

In the method presented here the specific action of uricase is made use of, so that the difference in color value before and after

* A brief preliminary report of this work has been published (1).

uricase action is a true measure of the uric acid content. It has also been found that by omitting the heating during color development the color intensity is more nearly directly proportional to the concentration of uric acid. Nevertheless, for the most accurate values in which appreciable differences in the uric acid content of the standard and the unknowns are compared, we find it necessary to make corrections based on empirically observed colorimeter readings over a wide range of uric acid concentration.

Reagents—

Uricase preparation. 1 pound of fresh, frozen beef kidney, which had previously been mechanically defatted and ground fine in a food chopper, was dehydrated with four samples of 500 cc. each of acetone over a period of 48 hours at 4°. 12 hours were allowed for each extraction. The tissue was then dried quickly at room temperature with the aid of a fan. The dry material was treated twice at room temperature with 500 cc. of benzene, the first treatment for 6 hours, followed by filtering, and a second treatment for 12 hours. The resulting material was dried quickly at room temperature, ground to 60 mesh with a mechanical mill or a mortar and pestle, and stored in a desiccator at 4°.

The activity of the powder was determined by following the rate at which it caused the disappearance of uric acid from a solution. Since the activity depends on the conditions used in the determination, those conditions are stated precisely. 1 gm. of uricase powder was suspended in 100 cc. of 0.125 per cent sodium carbonate, and 1 cc. of toluene was added. Quantities of 1 cc. of the well mixed suspension were incubated with from 10 to 20 mg. of uric acid dissolved in 10 cc. of 0.15 per cent lithium carbonate in a 125 cc. Erlenmeyer flask for 2 hours at 45°. ¹ At the end of this time 36 cc. of water, 1 cc. of 10 per cent sodium tungstate, and 2 cc. of $\frac{2}{3}$ N sulfuric acid were added. The uric acid was determined colorimetrically on the filtrate. In a typical experiment, 1 cc. of the 1 per cent suspension oxidized 4.85 mg. of uric acid, or 1 gm. of the powder oxidized 485 mg. of uric acid.

The activities found for this preparation compare favorably with those reported by Ro (10) and by Truszkowski and Gold-

¹ Ro (10) showed 45° to be the optimum temperature for uricase action.

manowna (11) for their preparations. The dry preparation was stable for a year.

Sodium tungstate. A 10 per cent solution was prepared with the usual precautions as to alkalinity. The sodium tungstate of Pfanstiehl was found satisfactory.

Sulfuric acid. Standard $N/12$ and $\frac{2}{3} N$ solutions.

Uric acid reagent (Koch modification) (12). 100 gm. of sodium tungstate (Pfanstiehl's best grade) were dissolved in 700 cc. of distilled water in a 2 liter, round bottom flask. 75 cc. of 85 per cent phosphoric acid were added and the mixture boiled gently under a reflux condenser for 24 hours. The resulting solution was decolorized by adding a few drops of bromine or 30 per cent hydrogen peroxide to the hot solution and boiling without the condenser for 10 minutes to remove the excess. The pale yellow liquid was diluted to 1 liter, filtered, and stored in a brown glass-stoppered bottle.

Urea-cyanide solution (8). 250 gm. of Merck's or Baker's best grade urea were dissolved in 700 cc. of water, filtered, and diluted to 1000 cc. This solution is stable for months when stored at refrigerator temperature. 2.5 gm. of Merck's or Baker's best grade sodium cyanide² were dissolved in 100 cc. of the 25 per cent urea solution as needed. This solution is stable for 2 to 3 days when stored in the refrigerator, but it should be discarded after that time.

Standard uric acid solution. A stock solution of uric acid in lithium carbonate, containing 1 mg. of uric acid per cc. of solution and preserved with formaldehyde, was prepared according to the directions given by Koch (12) for the Folin stock solution. For the standard solution, 1 cc. of this stock solution was diluted to 200

² Unfortunately different samples of sodium cyanide give different intensities of color with the same sample of standard uric acid and reagents. The picture is complicated still further by the fact that the variations in intensity with different cyanides and pure uric acid solutions are different from the variations observed with the same cyanide and blood filtrates—in extreme cases making a difference in the calculated value of uric acid of as much as a mg. per 100 cc. of blood. This emphasizes the necessity of using the same cyanide throughout any comparative study. The sodium cyanide now purchasable contains only 95 per cent sodium cyanide. It is possible that the preparation of a cyanide free from impurities would eliminate this difficulty.

cc. 1 cc. of this diluted solution contains 0.005 mg. of uric acid. The stock solution is stable, but the diluted standard should be freshly prepared at least every 3 days.

Procedure

Two blood filtrates were prepared: (a) a tungstic acid filtrate of whole blood, and (b) the same type of filtrate made with the same blood after the uric acid had been destroyed by uricase. For the untreated blood filtrate, 2 cc. of 10 per cent sodium tungstate were added to 2 cc. of blood placed in a 50 cc. centrifuge tube. To this mixture 16 cc. of $N/12$ sulfuric acid were slowly added. Then the mixture was centrifuged or filtered to obtain a clear filtrate. For the other filtrate, 2 cc. of blood were incubated in a water bath at $40-48^{\circ}$ for 2 hours with 50 mg. of the uricase powder, at which time the blood proteins and uricase were precipitated by adding 2 cc. of the 10 per cent tungstate and 16 cc. of $N/12$ sulfuric acid. A filtrate was obtained from this mixture by centrifuging or filtering.

For the color development, duplicate 5 cc. samples of the filtrate from the blood treated with uricase and duplicate samples of from 1 to 5 cc. of the untreated blood filtrate were placed in 1×8 inch test-tubes. Standards containing from 1 to 5 cc. of the standard solution were also prepared.³ The volume of liquid in all the tubes was adjusted to 5 cc. To these tubes 5 cc. of the fresh 2.5 per cent NaCN in 25 per cent urea were added from a long tipped microburette.⁴ Then 1 cc. of uric acid reagent was added slowly from a long tipped burette directly into the solution. The contents of the tubes were mixed well, and the tubes were stoppered and allowed to stand at room temperature for 3 hours for color development, after which the unknowns were compared in a colorimeter with the appropriate standard.

The apparent uric acid value obtained with the blood treated with uricase was subtracted from that of the untreated blood to give the true uric acid content.

³ Usually only 5 cc. samples of both filtrates and 1 and 3 cc. standards were needed.

⁴ The cyanide solution, the uric acid reagent, and the standard solution were stored in Koch microburettes ((12) p. 237) and measured directly from them.

Corrections for Lack of Parallelism between Color Intensity and Concentration of Uric Acid Present—In order to eliminate this

TABLE I

*Percentages by Which Calculated Uric Acid Must Be Adjusted to Correct for Lack of Parallelism of Color Intensity and Concentration, Given by Observed Colorimetric Readings with Standard Set at 20 Mm.**

Colorimeter reading	Percentage to be added		Colorimeter reading	Percentage to be subtracted	
	With 0.015 mg. standard†	With 0.005 mg. standard‡		With 0.015 mg. standard	With 0.005 mg. standard
<i>mm.</i>			<i>mm.</i>		
10.5-11.4	4.5	11.2	20.1-20.4	0.2	0.5
11.5-12.4	4.2	9.5	20.5-21.4	0.9	2.9
12.5-13.4	3.8	8.1	21.5-22.4	1.6	5.5
13.5-14.4	3.2	7.1	22.5-23.4	1.9	6.7
14.5-15.4	2.8	6.3	23.5-24.4	2.4	7.6
15.5-16.4	2.2	5.3	24.5-25.4	3.1	8.8
16.5-17.4	1.3	3.9	25.5-26.4	3.6	10.6
17.5-18.4	0.8	2.5	26.5-27.4	4.0	12.4
18.5-19.4	0.5	1.1	27.5-28.4	4.5	14.6
19.5-19.9	0.1	0.2	28.5-29.4	5.0	16.2
20.0-20.0	0.0	0.0	29.5-30.4	5.5	18.9

* An illustration of the use of the figures in the table follows: In a case in which the color produced with 5 cc. of blood filtrate was compared with that of a 0.015 mg. standard set at 20 mm., a colorimetric reading of 16.0 mm. was observed. By direct calculation, 3.75 mg. of uric acid per 100 cc. of blood are obtained, but to correct for lack of parallelism in color intensity this value must be corrected by adding 2.2 per cent ($0.022 \times 3.75 = 0.0825$); therefore, the value for this sample is ($3.75 + 0.08$) 3.83 mg. per 100 cc. of blood.

† The 0.015 mg. standard can be used in most analyses in which 5 cc. of filtrate of blood not treated with uricase are used. The same percentage corrections are applicable for use with from 0.010 to 0.020 mg. standards.

‡ The 0.005 mg. standard is usually needed with blood previously treated with uricase. The same percentages are applicable for corrections with standards of from 0.0025 to 0.0075 mg.

error and to reduce the number of standards needed for each determination, curves were developed for each standard used.

In the early colorimetric methods heat was applied to speed up the color development.⁵ We have found a greater deviation from the true proportionality when heat is included in the procedure, thus confirming in part the findings of Brown (15). Because of this discrepancy, the heat treatment was rejected in the method described here. When uric acid reduces the reagent at room temperature, the maximum error due to lack of proportionality is ± 5 per cent, even when readings deviate 8 to 10 mm. from that of the standard, and corrections can be made for this error.

In this study the calculated uric acid concentrations for both untreated and uricase-treated blood have been adjusted to the correct values by applying the corrections found necessary from these experimental curves. For convenience, the corrections are given in terms of percentages and are recorded in Table I. To correct the calculated uric acid concentrations, uric acid was added if the unknown reading was less, and subtracted if the reading was greater than that of the standard. The blank color value is proportionally greater when the concentration of uric acid present is less. Thus with the low standard used for blood treated with uricase the percentages necessary for corrections are larger than with the 0.015 mg. standard. For the results reported here, corrections were not made when colorimeter readings agreed within 2 mm. of that of the standard, and determinations were repeated with a more appropriate volume of filtrate when readings deviated more than 8 mm. from that of the standard.

Results

The reliability of the new method was tested by recovery studies on pure uric acid solutions and on human blood. The quantitative

⁵ The lack of parallelism between color intensity and concentration of uric acid in the Folin and Denis (2) method was noted in 1914 by Steinitz (13). In 1924 Cohen (14) suggested that this defect is due to the blank color of the reagents and gave a method for correcting mathematically for the blank of Benedict's (4) method. Brown (15) in 1926 showed that if heat is not applied while uric acid reduces a phosphotungstate, the color intensity is proportional to the concentration of uric acid. For some time these findings were ignored. Since heating is omitted in the latest methods of Folin (8) and of Newton (9), it is possible that this change of procedure, as well as the change of reagents, is responsible for the truer proportionality claimed for these methods than was observed with their earlier methods.

destruction of uric acid added to blood by incubation of the blood with uricase furnished strong evidence in favor of the procedure as an accurate method for determining uric acid present in blood.

Uric Acid Values on Pure Uric Acid Solutions—The direct colorimetric procedure was used in testing the concentrations of pure uric acid solutions containing from 1 to 6 mg. of uric acid per liter, which is the range covered by blood filtrates. With six unknowns made up in such quantities that duplicate determinations could be analyzed, as outlined in the procedure above, recoveries ranged from 100 to 101.6 per cent (percentage error from 0 to +1.6 per cent). With six unknowns, for which no duplicate analyses were possible, recoveries were from 97.2 to 105.1 per cent (percentage errors from -2.8 to +5.1 per cent). The average recovery for the twelve unknowns was 101 per cent. The errors in these unknowns are typical of those to be expected when the colorimetric procedure is applied to blood filtrates.

Uric Acid Content of Human Blood—The average uric acid value for 50 samples of human blood was 2 mg. per 100 cc. of blood with a range of from 1.04 to 3.83 mg. The apparent uric acid for these samples obtained by the direct procedure without uricase action, ranged from 1.82 to 4.60 mg. with an average of 3 mg. per 100 cc. of blood. The non-uric acid color value of these samples obtained by determinations after uricase had destroyed the uric acid averaged 1 mg. per 100 cc., ranging from 0.60 to 1.54 mg., when calculated as uric acid.

Substances Other Than Uric Acid Which May Contribute to Color Produced in Direct Colorimetric Procedure—The non-uric acid color value left after uricase has acted on the blood must be accounted for. Table II gives a list of substances that may contribute to this value. The data in Table II were arrived at by preparing solutions of glutathione, ascorbic acid, cystine, cysteine, glucose, tyrosine, and resorcinol in concentrations comparable to the maximum concentrations in which these substances are reported to occur in blood. These solutions were incubated with uricase at pH 9.5 for 2 hours at 45°, the uricase was precipitated with tungstic acid, and these filtrates, as well as the original solutions, were tested for their blank color values by the colorimetric uric acid procedure. Dilute solutions of the stock uric acid were used as standards, and the color formed was calculated as mg. of uric

acid. As will be seen from Table II, the color before and after uricase action in every case is approximately the same with the exception of glutathione. Schroeder and Woodward (16) have shown the presence of a hydrolytic enzyme for glutathione in kidney tissue. It is possible that the enzyme may be present in the kidney uricase preparation used in this study, the amino acids liberated by its action giving less color with the reagents than the original glutathione. If this be the case, it is an error that could be corrected by the use of a purer uricase. Resorcinol is included in the list, since early papers (17) gave the polyphenol

TABLE II

Substances That May Contribute to the Non-Uric Acid Color Value As Found in Blood after Uricase Action

Substance	Concentration, mg. per 100 cc. blood*	Calculated as mg. uric acid per 100 cc. blood†	
		Before uricase action	After uricase action
Glutathione.....	40	0.60	0.50
Glucose.....	100	0.08	0.08
Ascorbic acid.....	2	0.05	0.09
Resorcinol.....	5	0.27	0.27
Tryptophane.....	2 (NH ₂ -N)	0.04	0.04
Tyrosine.....	2 "	0.10	0.10
Cystine.....	2 "	0.10	0.11
Cysteine.....	2 "	0.15	0.13

* The solutions used contained the substances in concentrations comparable with the maximum concentrations in which they may appear in normal blood filtrates.

† All values are corrected by a blank value of 0.15 mg. per cent.

content of blood as 2 to 5 mg. per cent. Since a recent paper (18) suggests that the polyphenol content of blood is about 0.02 mg. per cent rather than the higher value, possibly polyphenols do not contribute to the non-uric acid color value of blood filtrates.

The substances found not to contribute to the non-uric acid color value are allantoin, alloxan, adenine, guanine, hypoxanthine, xanthine, adenylic acid, creatine, creatinine, and the uricase preparation. Ergothioneine undoubtedly contributes to this factor. Due to the unavailability of this substance and to lack of time for its preparation it was not studied.

Destruction of Uric Acid Present in Human Blood and of Uric Acid Added to Blood by Uricase—Kleinman (19) in 1934, using a combination of the direct colorimetric procedures of Benedict and Folin, reported that from 50 to 70 per cent of the uric acid present in whole blood was oxidized when incubated for 18 hours at 37° with a uricase preparation of pig liver. He considered the entire color produced by the direct procedure to be due to uric acid. As has been stated previously an average of two-thirds of the color produced with the reagents in this study with the blood filtrates is due to the presence of uric acid. That the fraction left after uricase action is not uric acid is evidenced by the fact that uric

TABLE III

Quantitative Destruction of Uric Acid Added to Human Blood When Incubated with Uricase

All figures are in mg. per 100 cc. of blood.

Sample No.	Original blood			Uric acid added	Blood + uric acid			
	Before uricase action	After uricase action	True uric acid		Before uricase action	After uricase action	Uric acid found	Theory for true uric acid*
1	3.00	0.97	2.03	4.0	6.39	0.97	5.42	6.03
2	3.24	0.79	2.45	4.0	6.40	0.80	5.60	6.45
3	4.21	1.10	3.11	5.0	8.93	1.10	7.83	8.11
4	3.42	0.78	2.64	4.0	7.15	0.91	6.24	6.64
5	3.00	0.81	2.19	4.0	6.10	0.82	5.28	6.19
6	3.33	0.89	2.44	16.0	19.02	0.90	18.12	18.44

* Percentage recoveries range from 83.7 for Sample 2 to 98.2 for Sample 6, with an average recovery of 91.3 for the six samples.

acid added to blood is quantitatively destroyed when incubated with uricase, leaving the same non-uric acid color value as before (see Table III). Blood incubated with uricase for 1, 2, 3, and 24 hour periods showed the same residual color value; thus ample time was allowed for oxidation of all the uric acid present.⁶

Recovery of Uric Acid Added to Human Blood—Recoveries of uric acid added in quantities of 2 to 16 mg. per 100 cc. of blood

⁶ The amount of uricase incubated with the blood in this method is sufficient to oxidize at least 100 times the amount of uric acid present in the blood; this excess was used in order to insure complete destruction of all uric acid present, and to speed up the action.

averaged 93.5 per cent in the sixteen samples shown in Tables III and IV. This low recovery can be accounted for in part by the method of protein precipitation. If the acid for protein precipitation was added slowly, as long as 20 minutes being allowed for the precipitation, recoveries were better. Uric acid added to blood filtrates was recovered with greater accuracy than when added to the blood directly.

It might be thought that the presence of ergothioneine and glutathione in the filtrates accounts in part for the low recoveries. In the Newton (9) method in which these two substances are

TABLE IV
Recovery of Uric Acid Added to Human Blood

Sample No.	Uric acid per 100 cc. blood				Per cent recovery
	Original blood	Amount added	Blood + uric acid		
			Theory	Found	
	mg.	mg.	mg.	mg.	
1	2.78	4.0	6.78	6.26	92.3
2	3.46	4.0	7.46	7.27	97.5
3	2.05	2.0	4.05	3.81	94.1
4	2.68	16.0	18.68	16.58	88.8
5	3.42	16.0	19.42	19.35	99.6
6	3.15	4.0	7.15	6.68	93.4
7	3.02	4.0	7.02	6.40	91.2
8	2.52	4.0	6.52	6.32	96.9
9	2.70	4.0	6.70	6.64	99.1
10	3.00	16.0	19.00	18.00	94.7
Average.....					94.8

precipitated from the filtrate before the uric acid determination, the recoveries average 90.8 per cent.

The destruction of uric acid by blood as a possible cause of the low recoveries was investigated and is discussed in the following paper (20).

DISCUSSION

The phosphotungstic acid colorimetric method for the determination of uric acid in blood is made specific by subtracting the chromogenic value of a filtrate prepared from the blood which

has had its uric acid destroyed by uricase from the chromogenic value of the untreated blood. The outstanding advantage of the method is its specificity. Uric acid can be determined on regular Folin-Wu tungstic acid filtrates of whole blood which contain other substances that reduce the reagent, since the uric acid is measured by its quantitative destruction by uricase.

The method is time-consuming—recovery studies showed that the extended interval allowed for reduction of the reagent was necessary—but this is offset by the large number of samples that can be analyzed simultaneously. The curves for correction of lack of parallelism of color intensity and concentration of uric acid reduce to a minimum the number of standards needed for accurate determinations.

SUMMARY

A new colorimetric method for the determination of uric acid in whole blood has been devised. The specificity of the method is based on the destruction of uric acid by uricase.

By this method an average chromogenic value for normal blood as found by the direct reduction of a phosphotungstate in terms of uric acid is 3 mg. per cent. Only two-thirds (about 2 mg. per cent) of this color is due to uric acid. The other third (1 mg. per cent) originates from the oxidation of other substances which are not destroyed by uricase.

The substances studied which may contribute to the non-uric acid color value of whole blood filtrates are glutathione, phenols, ascorbic acid, glucose, tyrosine, tryptophane, cystine, and cysteine.

Recoveries of uric acid from water solutions by the direct procedure ranged from 97 to 105 per cent and averaged 101 per cent. Recoveries of uric acid added to whole blood ranged from 85 to 99 per cent and averaged 93.5 per cent.

The reliability of the oxidation of uric acid by uricase as a measure of the uric acid content of whole blood is shown by the quantitative destruction of uric acid added to blood when it is incubated with uricase according to the method.

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APPLICATION OF THE URICASE METHOD TO THE STUDY OF CHANGES IN VITRO IN THE URIC ACID CONTENT OF CERTAIN MAMMALIAN BLOODS*

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The blood of mammals is generally considered to be free from uricase (2, 3). Nevertheless, evidence for a catalytic destruction of uric acid by blood was reported by Flatow (4) in 1926, by Gomolinska (5) in 1928, and by Rowińska (6) in 1930. Flatow, who used his own ferricyanide titration method (7) for the determination of uric acid, stated that all uric acid values for mammalian blood would be of the order of 10 to 20 mg. per 100 cc. of blood were it not for the presence in blood of a heat-stable catalyst which destroys uric acid so rapidly that by the time analyses are made (usually from 1 to 2 hours after the blood is drawn) an average value of 2 mg. per cent is observed with human blood and only traces with horse and sheep blood. This "oxidase," present in the red cells and in filtrates prepared from whole blood, was shown to act on added uric acid as well as that originally present in the blood. Neither Gomolinska (5) nor Rowińska (6) observed such drastic destruction of uric acid, but they agreed with Flatow that the erythrocytes contain a substance capable of destroying uric acid.

The presence in human blood of an oxidase such as that suggested above would mean that routine clinical analyses of blood for uric acid should be made at a definite time interval following the drawing of the blood, and could also account for the lack of complete recovery of uric acid added to blood.

Since studies with the mammals used routinely in experimental laboratories are of little value for interpreting purine metabolism of man, and since the bloods of these animals are generally thought

* A brief preliminary report of a part of this work has been published (1).

to contain uric acid in such low concentrations as to make determinations by isolation procedures tedious and by direct procedures unreliable, relatively little work has been reported on the uric acid content of these bloods. Therefore a study of these bloods for the presence of high concentrations of easily oxidizable uric acid-like substances, such as Flatow (7) reported for horse and sheep blood, might throw new light on purine metabolism in these animals.

Taking advantage of the specificity of the uricase method described in the preceding paper (8), we have investigated the blood of man, dog, rabbit, rat, guinea pig, and mouse for (a) the content of uric acid immediately after the removal of the blood from the animal, (b) the changes that occur in the uric acid content on incubation *in vitro*, and (c) the presence of agents causing the disappearance or the formation of uric acid.

EXPERIMENTAL

Effect of Human Blood on Uric Acid—Human blood was drawn, oxalated, and analyzed for uric acid immediately. The analyses were repeated at definite intervals as the blood incubated in the refrigerator. As will be seen from the data of a typical experiment shown in Table I, the uric acid content of the blood after it had stood for 24 hours and even 5 days corresponded with that found by the original estimation. Both values found by direct determinations, as well as the non-uric acid color values, remained constant for the duration of the experiment.

In these experiments the time of precipitation of proteins was taken as the time of analysis. Filtrates were prepared rapidly by centrifugation; they were sampled immediately, and the reagents for oxidation of uric acid were added as quickly as possible. This procedure is not rapid enough for the detection of any destruction that might have occurred in the first 15 minutes after the drawing of the blood, but the constancy of the values obtained after that time indicates that routine analyses of blood several hours after it is drawn still reveal the true picture of uric acid content.

Uric acid added to blood was recovered with the same accuracy after it had stood with the blood for a day as when determined immediately after being placed with the blood (Table II). It is not our aim in this paper to dispute the findings of Flatow but

rather to state that the action of an oxidase such as he and Gomolinska describe does not account for the lack of complete recovery of uric acid by the uricase method. The occlusion of uric acid during the protein precipitation still holds as the factor contributing most to this error. Undoubtedly, other factors contribute to the lack of recovery but for the present an average recovery of

TABLE I
*Uric Acid Content of Human Blood As Observed at Various Intervals
Following Removal from Body*

Time elapsed between drawing of sample and analysis	Mg. uric acid per 100 cc. blood		
	Before uricase action	After uricase action	True uric acid
10 min.....	3.28	0.98	2.30
20 "	3.35		2.35
35 "	3.16		2.16
2.5 hrs.....	3.13	1.00	2.13
24 hrs.....	3.28	1.00	2.28
5 days.....	3.16	0.87	2.29
8 "	3.00	0.89	2.11

TABLE II
*Recovery of Added Uric Acid from Human Blood, in Relation to Time
of Contact*

Sample No.	Interval uric acid was in blood	Mg. uric acid per 100 cc. blood				Percentage recovery
		Original blood	Uric acid added	Blood + uric acid		
				Theory	Found	
1	5 min.	3.03	4.0	7.03	6.51	92.6
	24 hrs.	3.12	4.0	7.12	6.61	92.8
2	5 min.	3.05	16.0	19.05	17.97	94.3
	24 hrs.	3.00	16.0	19.00	17.64	92.8

93.5 per cent was considered satisfactory, and the question has not been investigated further in this study.

Uric Acid Content of Dog Blood—For these experiments blood was drawn from the femoral vein of unanesthetized dogs, oxalated, and analyzed immediately. The analysis was repeated at various intervals as the blood remained in the refrigerator. In the first

experiments the blood was analyzed at 10 minute intervals for the 1st hour and at 30 minute and 1 hour intervals after that time. No high values were observed either when the blood was fresh or while it was stored in the refrigerator for several days.

In all the samples tested considerable color was produced with the reagents used in the procedure, but very little of the reduction of the reagent was caused by uric acid, since the color intensities produced with filtrates prepared both before and after uricase had acted on the blood were nearly the same.

The average figures given in mg. of uric acid per 100 cc. of blood for ten samples of dog blood were 2.00 (1.25 to 2.74) for untreated blood, 1.82 (1.18 to 2.53) for blood incubated with uricase, and 0.18 (—0.02 to 0.45) for the difference or the true uric acid. In no case was a uric acid content higher than 0.5 mg. per cent observed with a sample of dog blood.

The validity of these low figures as the uric acid content of dog blood was substantiated by the following findings: (a) Uric acid added to dog blood was destroyed when incubated with uricase during the procedure of the method, leaving the same non-uric acid color value as the original blood, and (b) added uric acid was recovered from dog blood with approximately the same accuracy as from human blood. Thus dog blood contains no uric acid-destroying agent or any other substances that prevent the detection of uric acid by the uricase method.

Uric Acid Formation in Rat Blood—The study of this animal seemed particularly worth while because uric acid was detected in the blood of rats by Folin and Morris (9) in 1913. For this work blood was drawn by heart puncture from normal adult white rats, oxalated, and pooled for analysis. The blood was sampled immediately and its proteins precipitated, the uric acid value being found to be near that of low values for human blood. The next sample of the same blood showed a considerably higher uric acid content and so did the succeeding ones (Table III). This was the opposite of what would have been expected from the findings of Flatow (4). Folin and Morris (9) made no statement as to time elapsed between the drawing of the rat blood for the experiments and the time of analyses. Blood drawn under sterile conditions and kept sterile for the duration of the experiment and that drawn without attempting to use sterile technique showed the

same increase in uric acid when the blood was allowed to stand. This increase was about 2 to 3 mg. per cent in 24 hours when the blood was stored in the refrigerator and about 7 mg. per cent in 8 days. Different samples of blood showed widely different original values for their uric acid content, but all showed the same increase with time. Uric acid was produced more rapidly in blood

TABLE III

Uric Acid Content of Rat Blood As Observed at Various Intervals Following Removal from Animal

Pooled samples of blood drawn with sterile technique, oxalated, and kept sterile.

Sample No.	Time elapsed between drawing of sample and analysis; additional treatment of blood	Mg. uric acid per 100 cc. blood		
		Before uricase action	After uricase action	True uric acid
1	None*	1.45	0.69	0.76
	2 hrs.	2.70	0.54	2.16
	7 "	3.14		2.60
	26 "†	3.80	0.43	3.37
	4 days	6.30	0.45	5.85
	8 "	8.21	0.57	7.64
2	1 hr.	1.98	0.86	1.12
	Incubated 3 hrs. at 40-48°	4.82	0.86	3.96
	3 days†	5.43	0.47	4.96
3	2 hrs.	2.45	0.65	1.80
	Remained 3 hrs. at 4°	2.58	0.65	1.93
	Incubated 3 " " 40-48°	4.82	0.65	4.17
	30 hrs.†	5.50	0.49	5.01

* Approximately $\frac{1}{2}$ hour elapsed while the samples were being drawn and pooled; the proteins were precipitated immediately after the whole sample was collected.

† The blood remained in the refrigerator.

incubated at 40-48°, the increase in 2 to 3 hours being comparable to that observed in blood standing 24 hours in the refrigerator. Uric acid formed more slowly in blood preserved in an ice bath at 4°. All uric acid formed in rat blood was destroyed when the blood was incubated with uricase, but the non-uric acid color value for the blood remained comparatively stable for a given sample of blood. The production of uric acid was not observed

in blood filtrates, the uric acid value of incubated filtrates remaining constant for the duration of the experiment.

The above findings indicate that some enzymatic action—either a decomposition of a complex or an oxidation of a precursor—is responsible for the formation of uric acid in rat blood. In 1915 Benedict (10) noted that incubation of beef blood at room temperature for 2 weeks raised the uric acid content from 0.5 to 7.0 mg. per cent. Later the uric acid was shown to arise from the decomposition of uric acid-*d*-riboside present in the blood (11). Combined uric acid was shown to be present in smaller amounts in human, horse, sheep, pig, dog, and chicken blood (12). An investigation of rat blood for the presence of a similar uric acid complex seemed the most logical procedure to follow, but attempts to increase the uric acid content of filtrates of rat blood by hydrolyzing some hypothetical nucleotide or nucleoside present in them failed.

Search for the presence of hydrolytic enzymes for the decomposition of conjugated uric acid was then abandoned in favor of a search for precursors and enzymes which might produce uric acid by oxidation. Since the less oxidized purines seemed to be the compounds most likely involved, they were studied as precursors. The purines were purchased from Hoffmann-La Roche, Inc., and were found of high purity according to the reactions of these compounds as given by Levene and Bass (13). The purines studied here do not reduce the reagents used in the uric acid determination either before or after incubation with or without uricase (8).

Xanthine, being one step less oxidized than uric acid, was the first purine studied. In this experiment 1 mg. of xanthine dissolved in 1 cc. of 0.01 *N* sodium hydroxide was incubated for 2.5 hours with 1 cc. of rat blood. The first sample tried showed an increase in the uric acid from 4 mg. to 40 mg. per 100 cc. of blood. In Table IV are recorded typical results obtained by the incubation of 1 mg. quantities of adenine, hypoxanthine, guanine, and xanthine with 1 cc. quantities of rat blood. Uric acid was produced in large quantities from both guanine and xanthine; in the case cited 1 cc. of blood produced 0.2 mg. of uric acid from 1 mg. of guanine and 0.3 mg. of uric acid from 1 mg. of xanthine in 4 hours at 40–48°. The control blood incubated with the same

amount of alkali as that in which the purines were dissolved also showed a slight increase in the uric acid content. Neither adenine nor hypoxanthine in the concentrations used in these experiments was changed to uric acid. That the blood contains enzymes for the transformation of these compounds to uric acid is discussed in the following paper (14).

Blood 8 days old still had the ability to oxidize xanthine to uric acid; therefore the enzymes present in blood which produce uric acid are fairly stable.

TABLE IV

*Uric Acid Produced in Rat Blood by Incubation with Purines**

All incubations were for 4 hours at 40–48°.

Treatment of blood	Mg. uric acid per 100 cc. blood	
	Before uricase action	True uric acid
Stood at room temperature 4 hrs.....	6.77	5.69†
Incubated with no additions.....	9.54	8.46
“ “ alkali.....	10.25	9.17
“ “ adenine.....	8.31	7.23
“ “ guanine.....	33.10	32.02‡
“ “ hypoxanthine.....	8.42	7.34
“ “ xanthine.....	40.50	39.42‡

* 1 cc. of blood was incubated with 1 mg. of the individual purines dissolved in 1 cc. of 0.01 N sodium hydroxide.

† The non-uric acid color value left after uricase action was 1.08 mg. of uric acid per 100 cc.

‡ When uric acid values were above 20 mg. per cent, only 0.5 cc. of blood filtrate was used for the determination.

In every case in which uric acid was produced in rat blood it was destroyed by uricase. The uric acid was destroyed when the filtrates from the various samples of blood were adjusted to pH 9.5 with sodium carbonate and allowed to incubate with the uricase powder, or when the uricase was added to the blood after uric acid had been formed in it by incubation with the purines. Uric acid was destroyed as fast as it was formed when blood acted on guanine or xanthine in the presence of uricase. In all of these cases the non-uric acid color value left after uricase action was essentially the same as that of the original blood (Table V).

TABLE V

*Quantitative Destruction by Incubation with Uricase of Uric Acid
Formed in Rat Blood*

All incubations were for 2.5 hours at 40–48°.

Treatment of blood before analysis*	Mg. uric acid per 100 cc. blood	
	Direct determin- ation	True uric acid
A. No further incubation.....	9.43	8.32†
B. Incubated with uricase‡.....	1.11	
C. " " no additions.....	10.41	9.30
D. " " alkali.....	10.86	9.75
E. " " " and uricase.....	1.17	
F. " " adenine§.....	10.75	9.64
G. " " guanine§.....	30.30	29.19
H. " " " § and uricase.....	1.11	
I. " " " , § followed by incubation with uricase.....	1.13	
J. Incubated with hypoxanthine§.....	11.36	10.25
K. " " xanthine§.....	35.50	34.39
L. " " " § and uricase.....	1.08	
M. " " " , § followed by incubation with uricase.....	1.14	
N. Filtrate of original blood Sample A adjusted to pH 9.5 and incubated with uricase 	1.08	
O. Filtrate of Sample G adjusted to pH 9.5 and in- cubated with uricase 	1.10	
P. Filtrate of Sample J adjusted to pH 9.5 and incu- bated with uricase 	1.11	
Q. Filtrate of Sample K adjusted to pH 9.5 and incu- bated with uricase 	1.10	

* The blood had remained in the refrigerator for 4 days before being used for this experiment.

† An average value of 1.11 mg. per cent was subtracted in each case for the non-uric acid color value.

‡ The quantity of uricase used in all cases was 25 mg. except for Samples H, I, L, and M, in which cases 100 mg. of uricase were used.

§ 1 cc. of blood was incubated with 1 mg. of purine dissolved in 1 cc. of 0.01 N sodium hydroxide.

|| The uricase was reprecipitated with tungstic acid before the uric acid was determined.

In preliminary experiments on the distribution of xanthine oxidase between the different phases of the blood, both phases were

shown to be active in converting xanthine into uric acid. In a typical experiment in which 1 cc. quantities of plasma and cells from oxalated blood were incubated for 3 hours at 45° with 1 mg. quantities of xanthine, 23.0 mg. per cent of uric acid were formed from the added xanthine in the plasma and 13 mg. per cent of uric acid in the cells. The method of separation of the two phases of blood (whether by centrifugation without an anticoagulant except oil, or by centrifugation of a pooled oxalated sample, or by centrifugation of a clotted sample) appeared to have little effect on the distribution. The non-uric acid color value of the cells was found to be higher than that of the plasma but uric acid was formed in both phases of the blood as it incubated with no additions or with xanthine added. More detailed work is required before conclusions can be drawn as to which phase is more potent in the enzyme action.

We conclude that the blood of white rats contains very little uric acid as it is taken from the animal, but it contains enzymes which, on the exposure of the blood to air, bring about a rapid formation of uric acid from precursors present in the blood. The velocity of the action is increased by incubating the blood at 40–48°, and retarded by cooling the blood to 4°. The enzymes which are fairly stable while in the blood are removed or destroyed in the precipitation of the blood proteins by tungstic acid. The addition of guanine or xanthine to the blood before the incubation so significantly increases the amount of uric acid formed as to establish the presence of guanase or xanthine oxidase in rat blood. The following paper deals with a detailed study of the uric acid-producing enzymes present in rat blood, including mechanisms of inhibition and acceleration of their action.

Absence of Xanthine Oxidase from Human and Dog Blood—In order to have additional evidence that xanthine oxidase in rat blood forms uric acid in this blood as it is incubated, human and dog bloods were tested for the presence of this enzyme. Just as human and dog blood showed no formation or destruction of uric acid on incubation at room temperature, or in the refrigerator, so their uric acid content did not change when these bloods were incubated with nothing added, with alkali, or with the purines used with rat blood.

The sample of human blood used for following the change in

uric acid with time shown in Table I had a uric acid content of from 2.17 to 2.41 mg. per cent when incubated for 2.5 hours at 40–48° with no additions or with alkali or purines added, and a sample of dog blood under like conditions showed a uric acid content of from -0.08 to $+0.09$ mg. per cent.¹ In no case did the incubations cause the chromogenic value of the filtrates of untreated or uricase-treated blood to vary more than the variations recorded in Tables I and VI for these samples. The possibility that uric acid was formed in these bloods as they were incubated and that it was destroyed by some oxidase present as fast as it formed is ruled out by the previously stated finding that uric acid added to both human and dog blood can be recovered. Thus xanthine oxidase is not present in human or dog blood. The observation that this enzyme is absent from human blood confirms a similar finding of Cole, Ellet, and Womack (15).

Uric Acid-Producing Enzymes in Blood of Other Rodents—Since the rat appeared to be unique in carrying xanthine oxidase in its blood, the blood of other rodents seemed worthy of investigation. Blood from rabbits, guinea pigs, and mice was drawn by heart puncture and oxalated as in the experiments with rats.² The blood was sampled at definite intervals for the uric acid determination. Blood of individual rabbits was used, but with guinea pigs and mice it was necessary to use pooled samples. No attempt was made in these cases to keep the blood sterile, since experiments with rat blood had shown this to be an unnecessary precaution.

Rabbits were found to be like dogs in that they contain very little uric acid and no xanthine oxidase in their blood (Table VI). Uric acid added to this blood was recovered with an accuracy of 94 per cent and was quantitatively destroyed by incubation with uricase. This justifies the use of the uricase method in studying the uric acid content of rabbit blood. An insufficient number of samples of rabbit blood was analyzed to permit generalization as to the normal range of uric acid content.

In contrast to the rabbit, guinea pig blood showed reactions identical with those for rat blood. There was a rapid formation

¹ Additional data illustrating this point are recorded in Table VI, in which all the bloods studied are compared.

² We are indebted to Mr. Donald Otway for drawing the blood from mice, rats, and guinea pigs for use in this study.

of uric acid as the blood was allowed to incubate at room temperature or in the refrigerator, the action being accelerated at 40–48°.

TABLE VI

Changes in Uric Acid Content of Human Blood and Blood of Dogs, Rabbits, Rats, Guinea Pigs, and Mice When Incubated with and without Addition of Purines

Time elapsed between drawing of sample and analysis; additional treatment of blood*	Mg. uric acid per 100 cc. blood†					
	Human	Dog	Rabbit	Rat	Guinea pig	Mouse
Less than 30 min.....	2.50	0.38	0.48	0.55‡	1.04	
2 to 3 hrs.....	2.50	0.17	0.68	1.74		0.19
24 hrs.....	2.40	0.08		3.74	2.60	
4 to 5 days.....	2.60	0.45	0.83	6.26	3.85	7.08
7 " 12 "			0.75	9.19		
Incubated with no additions§	2.63	0.17	0.74	5.60	2.60	1.35
" " alkali.....	2.67	0.24	0.62	6.30	2.48	0.89
" " adenine 	2.52	0.19	0.66	4.50	2.00	
" " guanine 	2.40	0.16	0.72	20.50	21.50	
" " hypoxanthine ...	2.60	0.13	0.64	5.10	1.10	0.20
" " xanthine 	2.53	0.21	0.74	50.00¶	27.88	2.07

* The blood remained in the refrigerator except when being sampled for analyses.

† Each sample of blood used here showed considerably higher calculated uric acid values by the direct procedure. The average non-uric acid color values for these blood specimens expressed as mg. of uric acid per 100 cc. of blood were as follows: human 1.6, dog 1.8, rabbit 1.8, rat 1.2, guinea pig 2.0, and mouse 2.0 for fresh blood and 1.3 for blood 4 days old.

‡ This was the lowest value of uric acid observed with any sample of rat blood; usually much more had formed before the filtrate could be made.

§ All incubations were for 2 to 3 hours at 40–48°. In every case the incubation of the blood alone and with the purines was started when the blood was less than $\frac{1}{2}$ hour old except in the case of the rat blood; this particular sample of rat blood was 32 hours old with a uric acid content of 5 mg. per cent when the incubation experiment was started.

|| 1 cc. of blood incubated with 1 mg. of purine dissolved in 1 cc. of 0.01 N sodium hydroxide.

¶ This was the highest uric acid content observed in any experiment under the given conditions.

Guanase and xanthine oxidase were both found in the blood, since large amounts of uric acid were found after incubation of guanine and xanthine with samples of guinea pig blood (Table VI). Hypo-

xanthine, when used in as small a quantity as 0.1 mg. per 1 cc. of blood, was also oxidized to uric acid, and excess hypoxanthine caused an inhibition of the enzyme. A discussion of the results from similar experiments on rat blood is given in the paper which follows.

Preliminary experiments with mouse blood showed that uric acid was produced more slowly in it than in rat or guinea pig blood when the blood was freshly drawn, but considerable uric acid was formed in the mouse blood by the time it was 4 days old (Table VI). In this case the incubation with xanthine caused much less uric acid to form than was observed under similar conditions with rat or guinea pig blood, and incubation with alkali decreased the formation. It is possible that the increased uric acid in this blood has its origin in some precursor other than xanthine. Just as with rat blood the enzymes responsible for uric acid formation in mouse blood are removed or destroyed in the precipitation of the blood proteins by tungstic acid, for no increase in uric acid was observed in filtrates which had remained in the refrigerator for 6 days. The blood of mice will have to be investigated further before conclusions can be made as to the uric acid-forming enzymes present in it.

*Comparison of Changes Observed in Uric Acid Content of
Certain Mammalian Bloods*

In a study reported in 1925 Bulmer, Eagles, and Hunter (16) concluded that there is a substance other than uric acid in the blood of the rabbit, dog, cat, guinea pig, and ox which interferes with the direct determination of uric acid in these bloods. The present study shows that by taking advantage of the specificity of uricase, a clearer picture of the uric acid content of the whole blood of different mammals is obtained.

With the uricase method it has been possible to divide the mammalian bloods studied into three groups: (a) human, which contains 2 to 3 mg. per cent of true uric acid but which appears by the direct procedure to contain an additional 1 mg. per cent; (b) dog and rabbit, which contain very little uric acid (0 to 0.7 mg. per cent) but by the direct procedure appear to contain a considerable quantity (2 to 2.5 mg. per cent); and (c) rat and guinea pig, which contain little uric acid (0.5 to 1 mg. per cent) when freshly drawn,

but in which uric acid is formed rapidly as the blood stands.³ Since incubation of rat or guinea pig blood with guanine or xanthine produces high concentrations of uric acid, it may be concluded that the uric acid in these bloods is formed by guanase and xanthine oxidase. No evidence has been found for the presence of these enzymes in either human, dog, or rabbit blood.

Table VI records comparative results for the different types of blood studied. The figures recorded in Table VI were obtained from data of typical experiments not previously included in this paper. The values for dog and rabbit blood are similar to those found by Brown (17) and by Bulmer, Eagles, and Hunter (16) with isolation procedures. When the latter group (16) confirmed the finding of Benedict (10) that free uric acid forms in beef blood *in vitro*, they followed *in vitro* changes in human and rabbit bloods. The observation in the present paper that no detectable uric acid is formed on incubation of human or rabbit blood confirms a like finding of the above group (16). Bulmer, Eagles, and Hunter also report a value (0.7 mg. per cent) for the uric acid content of fresh guinea pig blood similar to that found here, but they did not follow *in vitro* changes in the blood of the guinea pig or of the dog, and rat blood was not included in their study.

Although xanthine oxidase appears in some tissue of each of the mammals studied here (18), it has been found thus far in the blood of only the rat and guinea pig. Isolated tissues of the rat have been shown to be exceedingly potent in xanthine oxidase (19, 20), rat liver (21) and rat kidney (22) having served as sources of the enzyme for recent studies. In none of these studies was blood included among the tissues investigated. The finding of xanthine oxidase in the plasma as well as in the cells suggests that the blood may pick it up from some of the numerous tissues in which it occurs. The kidney appears to be a likely source, since the kidney of the guinea pig as well as of the rat contains this enzyme (22), while the kidneys of the other mammals used in this study have been reported to be free from the enzyme (18). This does not rule out the possibility that the blood as a tissue itself may form the enzyme.

³ Mouse blood is not included in this grouping because insufficient data were collected with it to warrant its classification here.

SUMMARY

Destruction of uric acid by blood has been reported at various times. In the present study, however, no destruction of uric acid by human, dog, rabbit, rat, guinea pig, or mouse blood was observed.

The uric acid content of human, dog, and rabbit blood was found to be constant when oxalated samples were stored in the refrigerator for several days, or incubated for several hours alone or with adenine, guanine, hypoxanthine, or xanthine.

The uric acid content of rat blood and guinea pig blood, although very low when removed from the animal, was found to increase rapidly on standing. No evidence was found for the presence of a uric acid complex in these bloods which might account for the observed uric acid formation. The production of uric acid in these two types of blood was accelerated by incubation of the blood with guanine, xanthine, or low concentrations of hypoxanthine. Therefore, the blood of rats and of guinea pigs contains guanase and xanthine oxidase.

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A STUDY OF XANTHINE OXIDASE OF RAT BLOOD

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The discovery of xanthine oxidase in rat blood (1), in addition to raising the questions of its distribution in the different phases of the blood and its presence in other bloods (2), suggested the following questions: (a) how does quinimine, known to inhibit xanthine oxidase in rat liver, affect the uric acid production in this blood; (b) is the formation of uric acid in rat blood inhibited by cyanide, and, if so, what is the effect of methylene blue on this inhibition; (c) is the xanthine oxidase found here an enzyme that oxidizes xanthine but not hypoxanthine; (d) why does addition of adenine and hypoxanthine retard, rather than accelerate, the formation of uric acid in the blood; and (e) is adenase, as well as guanase, present in rat blood?

The experiments recorded here were performed to answer these questions. The blood was incubated aerobically under various conditions and the amount of uric acid formed, as determined by the uricase method (3), was used as a measure of xanthine oxidase activity.

Uric Acid Formation in Rat Blood As Affected by Quinimine, Cyanide, and Methylene Blue

Effect of Quinimine—Recently Bernheim and Bernheim (4) demonstrated a specific inhibition of xanthine oxidase of rat liver by the quinoid form of *p*-aminophenol. In the present study quinimine¹ was found to have little effect on the amount of uric acid produced in rat blood incubated without added substrates

¹ The Eastman product of *p*-aminophenol was twice recrystallized from alcohol, and a water solution of it warmed in air to convert it into the quinoid form (4).

but to decrease markedly the formation of uric acid from added xanthine (see Table I). This inhibition by quinimine adds evidence that the enzyme which produces uric acid in rat blood is xanthine oxidase. The greater inhibition observed when xanthine is added to blood, as compared to blood alone, is a quantitative difference, which does not exclude xanthine as the substrate in both cases. Perhaps quinimine more concentrated than 0.0002 M would cause a greater percentage inhibition. It was impossible to use more quinimine here, however, because more concentrated solutions reduced the uric acid reagent. The amount used inter-

TABLE I

Uric Acid Content of Rat Blood, Uric Acid Formed on Incubation of Blood Alone and with Added Xanthine in Presence and in Absence of Quinimine, and Percentage Inhibition of Xanthine Oxidase Action by 0.0002 M Quinimine

Sample No.	Treatment of blood	Mg. uric acid per 100 cc. blood				Per cent inhibition
		Without quinimine		With 0.0002 M quinimine		
		Found	Formed	Found	Formed	
1	No incubation	5.71				
	Incubated with alkali*	9.17	3.46	8.92	3.21	7.2
2	No incubation	8.32				
	Incubated with xanthine†	34.39	26.07	16.50	8.18	68.6
3	No incubation	4.58				
	Incubated with xanthine†	26.52	21.94	11.50	6.92	68.5

* The incubation for Sample 1 was for 4 hours at 40–48°.

† 1 cc. of blood was incubated 2.5 hours at 40–48° with 1 mg. of xanthine dissolved in 1 cc. of 0.01 N sodium hydroxide.

ferred in no way with the uric acid determinations, which confirms the finding of Bernheim and Bernheim (4) that the action of uricase is not inhibited by quinimine.

Effect of Cyanide—The xanthine oxidase system, contrary to the other dehydrogenase systems, is very sensitive to cyanide. Dixon and Keilin (5) explained this peculiar type of inhibition—which works slowly, is irreversible, and affects the methylene blue reduction as well as the oxygen uptake—on the basis of a destruction of the enzyme by a chemical union of it with the cyanide, and showed that the inhibition was prevented by the presence of

the substrate. Several authors (6, 7) have differed with Dixon and Keilin as to the mechanism of the cyanide inhibition in this dehydrogenase system which has been shown to be independent of an iron-containing catalyst (8, 9). Our experiments with cyanide and with cyanide plus methylene blue were carried out in order to have more confirmatory evidence that uric acid formation in rat blood is brought about by xanthine oxidase rather than by some other factor.

TABLE II

Uric Acid Content of Rat Blood, Uric Acid Formed on Incubation of Blood Alone and with Added Purines in Presence and in Absence of Cyanide, and Percentage Inhibition of Xanthine Oxidase Action by 0.005 M KCN

All incubations were for 4 hours at 40–48°.

Treatment of blood	Mg. uric acid per 100 cc. blood				Per cent inhibition
	Without cyanide		With 0.005 M KCN*		
	Found	Formed	Found	Formed	
No incubation.....	5.71		5.71		
Incubated with alkali.....	9.17	3.46	5.82	0.11	96.8
“ “ guanine†....	32.02	26.21	12.27	6.56	75.0
“ “ xanthine†....	39.42	33.65	10.82	5.11	84.8

* In these uric acid determinations the action of uricase on the uric acid of the blood containing the cyanide was inhibited only slightly. Perhaps the hemoglobin of the blood protected uricase from complete inhibition (10).

† 1 cc. of blood* incubated with 1 mg. of purine dissolved in 1 cc. of 0.01 N sodium hydroxide.

In Table II are recorded data of a typical experiment in which part of a pooled sample of blood was incubated with 0.005 M potassium cyanide and another part without cyanide, both in the presence and absence of added guanine or xanthine. It will be seen that there is an almost complete inhibition of uric acid formation in the blood containing no added substrates and 75 and 85 per cent inhibition in the cases in which guanine or xanthine was present. A typical sample of rat blood to which 0.01 M KCN was added immediately after the blood was drawn from the animal contained 1.70 mg. per cent of uric acid and this value did not change

as the blood remained in the refrigerator for 24 hours, but when it was incubated for 3 hours at 40–48° the uric acid was lowered to 0.8 mg. per cent.

Experiments similar to these just described were repeated with methylene blue as well as cyanide present (see Table III). It will

TABLE III

Uric Acid Content of Rat Blood When Incubated with Cyanide, with Methylene Blue, and with Cyanide Plus Methylene Blue in Presence and Absence of Xanthine or Hypoxanthine

Sample No.	Treatment of 1 cc. of blood*	Mg. uric acid per 100 cc. blood			
		With no additions	With 0.0001 M methylene blue†	With 0.01 M KCN	With 0.01 M KCN + 0.0001 M methylene blue
(1)	(2)	(3)	(4)	(5)	(6)
1a	No incubation	5.54	5.60	5.72	5.91
1b	Incubated with 1 cc. 0.005 N NaOH	7.49	7.76	5.32	3.34
1c	“ “ alkali and 0.005 M KCN	5.54			
1d	“ “ “ “ 0.02 “ “	5.23	3.29		
2a	No incubation	8.75‡			
2b	Incubated with 1 cc. 0.005 N NaOH	10.90	11.20	8.75	6.70
2c	“ “ 1 mg. xanthine	24.90	37.83	8.80	7.73
2d	“ “ 1 “ hypoxanthine	8.43	11.31		
2e	“ “ 0.5 mg. hypoxanthine	9.05	16.14		
2f	“ “ 0.25 “ “	13.18	38.21		
2g	“ “ 0.10 “ “	19.85	22.50	8.43	6.28

* All incubations were for 3 hours at 40–48°. 1 cc. of blood was incubated with 1 cc. of alkali or with purine dissolved in 1 cc. of sodium hydroxide.

† Methylene blue in 0.0001 M concentration interfered in no way with the uric acid determinations; it had no effect on the action of uricase, and was precipitated with the blood proteins, thus preventing any interference during the color production of the determination.

‡ This sample stood at room temperature 10 hours before being used for the experiment.

be noted from Samples 1a and 1b that again a decrease in the uric acid was observed when the blood was incubated with 0.01 M KCN (5.54 mg. without incubation changed to 5.32 mg. with incubation). With 0.005 M KCN the value remained the original 5.54 mg. per cent, but when 0.02 M KCN was used for the incubation the uric acid decreased to 5.23 mg. per cent.

Methylene blue not only did not prevent the inhibition, but actually caused the uric acid content to be decreased still further to 3.34 mg. per cent with 0.01 M KCN and 3.29 mg. per cent with 0.02 M KCN. The same type of results was observed with xanthine (see Sample 2c) and later also with hypoxanthine (see Sample 2g) added to the blood. One may generalize that cyanide prevents uric acid formation from xanthine and that methylene blue plus cyanide, rather than preventing this cyanide inhibition, actually decreases the uric acid below that originally present. It will be noted that methylene blue alone increases the uric acid values (see Column 4). The mechanism of action of cyanide, methylene blue, and cyanide plus methylene blue will be discussed later.

Formation of Uric Acid from Hypoxanthine

Effect of Concentration and Time—Since all preparations of xanthine oxidase that have been reported in the literature oxidize both hypoxanthine and xanthine, the presence of an enzyme in rat blood that oxidizes only xanthine seemed unlikely, but under the conditions of the experiments reported in the preceding paper (2) this appeared to be true. For convenience the purines used in this study were made up in such concentrations that 1 mg. of purine was dissolved in 1 cc. of 0.01 N sodium hydroxide; and because of the scarcity of rat blood, only 1 cc. of blood was incubated with 1 cc. of the purine solutions for the individual tests. Thus in these experiments, if all the purine added had been changed to uric acid, it would have been possible to have uric acid values for blood as high as 100 mg. per 100 cc.

In most enzyme reactions, an increase in substrate concentration increases the rate of change of the substrate, but such is not the case with xanthine oxidase. Dixon and Thurlow (11), using the Thunberg technique, showed that high concentrations of substrate (xanthine as well as hypoxanthine) block the enzyme surface and prevent free access of the hydrogen acceptor to the activated substrate for oxidation. Booth (12) showed that concentrations of hypoxanthine of about 0.007 M inhibit the action of xanthine oxidase aerobically as well as anaerobically. Since this is approximately the concentration used here, inhibition by excess substrate suggested itself as a possible explanation for the lack of formation of uric acid from hypoxanthine. The critical concentration which

causes inhibition depends upon the concentration of enzyme (11), and there was no way of predicting the concentration of xanthine oxidase that was present in the rat blood. It appeared that oxidation was taking place in all samples incubated with hypoxanthine, for the blood became dark in the same way as when xanthine was being oxidized, but with hypoxanthine no increase in uric acid was observed. These facts suggested that hypoxanthine as well as xanthine would be oxidized to uric acid if sufficient time were allowed for the diffusion of oxygen from the air into the reaction mixture, or if the time were kept constant and the amount of substrate decreased.

In Table IV are recorded results of an experiment in which the hypoxanthine concentration was decreased and the time allowed for oxidation was increased. Tests F, G, H, and I show that even though the time allowed for incubation is kept at 2.5 hours, as the concentration of hypoxanthine is lowered from 1 mg. per cc. of blood (which causes a complete inhibition of the formation of uric acid) to 0.1 mg., the inhibition is not only removed but the hypoxanthine is quantitatively converted into uric acid. Thus xanthine oxidase of rat blood oxidizes hypoxanthine as well as xanthine. Tests G, K, and L also show that as the time allowed for oxidations is increased more of the hypoxanthine is oxidized to uric acid. When 1 mg. quantities of hypoxanthine were incubated with 1 cc. quantities of blood, even for 24 hours, very little of it was converted into uric acid (see Table VI, Samples 2i and 2j).

That xanthine, in the concentration used, for all the previous experiments was also too concentrated for the maximum yield of uric acid is seen in the data for Tests M to Q. In Test O more uric acid was produced in 2.5 hours from 0.25 mg. of xanthine incubated with 1 cc. of blood than from 1.0 mg. of xanthine. Longer incubation with 1 mg. of xanthine produced more uric acid (Tests P and Q).

Effect of Methylene Blue on Production of Uric Acid from Hypoxanthine—Wieland and Mitchell (13) observed that oxygen absorption from air by a mixture of hypoxanthine and xanthine oxidase was increased by methylene blue; and Reindel and Schuler (14) showed that rat kidney causes the oxidation of hypoxanthine to uric acid when methylene blue is added to the system. These two findings and the finding in this study that the oxidation

of xanthine was considerably accelerated by the presence of methylene blue led to the study of the effect of methylene blue on

TABLE IV

Uric Acid Content of Rat Blood When Incubated with Different Concentrations of Hypoxanthine or Xanthine for Different Lengths of Time, and Percentage of Substrate Converted into Uric Acid

Test	Additions to 1 cc. blood			Time of incubation†	Mg. uric acid per 100 cc. blood		Percent- age of substrate converted into uric acid§
	Substrate		0.005 N NaOH		Found present	Formed from added substrate‡	
	Purine*	Amount					
		mg.	cc.	hrs.			
A	None	0.00	0.00	0.0	4.90		
B	"	0.00	0.00	2.5	5.20		
C	"	0.00	1.00	2.5	5.56		
D	"	0.00	1.00	11.0	6.00		
E	"	0.00	1.00	23.0	7.00		
F	Hypoxanthine	1.00	0.00	2.5	5.49	-0.07	0.0
G	"	0.50	0.50	2.5	8.56	3.00	4.9
H	"	0.25	0.75	2.5	17.42	11.86	38.4
I	"	0.10	0.90	2.5	17.62	12.06	97.6
J	"	0.10	0.00	2.5	17.52	11.96	96.8
K	"	0.50	0.50	11.0	16.42	10.42	16.9
L	"	0.50	0.50	23.0	17.72	10.72	17.4
M	Xanthine	1.00	0.00	2.5	28.04	22.48	20.3
N	"	0.50	0.50	2.5	32.25	26.69	48.3
O	"	0.25	0.75	2.5	28.76	23.20	84.0
P	"	1.00	0.00	11.0	50.12	44.12	39.9
Q	"	1.00	0.00	23.0	55.42	48.42	43.8

* The hypoxanthine and xanthine were dissolved in 0.01 N sodium hydroxide so that 1 cc. of the solution contained 1 mg. of purine.

† The incubations of 2.5 and 11 hours were carried out at 40-48°; the additional incubation to 23 hours was at 38-40° with toluene added as a preservative.

‡ The figures in this column were obtained by subtracting the amount of uric acid found present when the blood had incubated without the addition of purines from that found when the blood had incubated with purines.

§ The percentages were calculated on the basis that 100 mg. of hypoxanthine added to 100 cc. of blood could theoretically yield 123.5 mg. of uric acid if it were all oxidized, and that 100 mg. of xanthine under the same conditions could yield 110.5 mg. of uric acid per 100 cc. of blood.

the formation of uric acid from hypoxanthine. In Table III, Sample 2 (Columns 3 and 4), are recorded data of a typical ex-

periment showing the effect of methylene blue on the formation of uric acid in rat blood. Sample 2c shows that the formation of uric acid from xanthine is markedly increased by methylene blue. Methylene blue prevents the decrease in uric acid usually observed when blood is incubated with high concentrations (1 mg. per cc.) of hypoxanthine (see Sample 2d) and greatly accelerates the formation of uric acid from the lower concentrations of hypoxanthine. Sample 2f should be noted particularly, since only 2.28 mg. (13.18 — 10.90) of uric acid were formed from 25 mg. of hypoxanthine without methylene blue, but when methylene blue catalyzed the reaction, 27.01 mg. (38.21 — 11.20) of uric acid were formed. The theoretical yield of uric acid is 30.9 mg. Since the ratio of concentration of methylene blue to purine is 1:36, the mechanism of the action of methylene blue must be catalytic.

Effect of Mixed Substrates—Reindel and Schuler (14) suggested that all hypoxanthine in the system must be changed to xanthine before xanthine oxidase will catalyze the oxidation of xanthine to uric acid. In Table V are recorded data on mixed substrates which appear to support this hypothesis. Samples 1f and 2f show that 1 mg. quantities of hypoxanthine together with 1 mg. of xanthine almost completely prevent the formation of uric acid from the xanthine. Hypoxanthine also prevents the formation of uric acid from guanine (Sample 1h). When the concentration of substrate (hypoxanthine plus xanthine) is halved, as in Sample 2i, some uric acid is formed from the added substrates, for sufficient time has been allowed for oxidation of this amount of hypoxanthine (Sample 2g). Adenine, like hypoxanthine, inhibits formation of uric acid from xanthine but to a lesser degree. This will be discussed later.

Inhibition and Acceleration of Formation of Uric Acid in Rat Blood

Of the facts that have been presented here the following deserve interpretation: (a) xanthine added to rat blood in all concentrations studied causes an increase in uric acid, but in 2.5 hours lower concentrations (0.25 mg. per cc. of blood) cause greater absolute increases than higher concentrations (1 mg.); (b) low concentrations of hypoxanthine (0.1 mg. per cc. of blood) are quantitatively converted into uric acid even in short incubation periods (2.5 hours), while high concentrations of hypoxanthine (1 mg.) mark-

edly inhibit uric acid formation from naturally occurring or added precursors of uric acid; and (c) while methylene blue accelerates the oxidation of either xanthine or hypoxanthine to uric acid in rat blood and cyanide inhibits this formation of uric acid, a mixture of methylene blue and cyanide causes a decrease in the uric acid below that originally present.

Although inhibition of the action of xanthine oxidase by excess substrate and by cyanide has been interpreted on the basis of a specific combination (11) or a specific inactivation (5) and our data are not in disagreement with this point of view, there are only a few of the facts presented which demand such an interpretation. The most striking of these is stated in (a) above; namely, that more uric acid is formed by the incubation of 0.25 mg. of xanthine with 1 cc. of blood than is formed in the same time from 1 mg. (see Samples M, N, O of Table IV). Since reduction of uric acid is not possible here, blocking the enzyme surface by excess substrate remains as the most reasonable explanation for the observed inhibition by the higher concentration of xanthine. Most of the other findings presented here can be interpreted better in terms of a balance between the oxidizing action of the oxygen, methylene blue, and uric acid, and the reducing action of the hypoxanthine, xanthine, and cyanide.

The concentrations of the various oxidizing and reducing agents are given in micromoles per 2 cc. of diluted blood (mM per liter would be half of these values): 1 mg. of xanthine, 6.6 micromoles; 1 mg. of hypoxanthine, 7.4 micromoles; cyanide, 20 micromoles; methylene blue, 0.2 micromole; dissolved oxygen, 0.4 micromole (15); oxygen, bound as oxyhemoglobin, about 8 micromoles, assuming 8 mM per liter as the oxygen capacity of the hemoglobin of the original blood. In addition, under aerobic conditions, an indefinitely great reservoir of atmospheric oxygen is present, the availability of which will vary with the autoxidizability of the reducing agents in the system, and with the duration of the incubation.

From the quantitative data just given it is seen that 1 mg. of hypoxanthine (7.4 micromoles) is in great excess of the dissolved oxygen (0.4 micromole) and nearly equivalent to the total oxygen held as hemoglobin (8 micromoles). Since the hypoxanthine-xanthine system has a more negative E_0 value than the xanthine-

TABLE V

Uric Acid Content of Rat Blood after Incubation with Individual or Mixed Substrates of Adenine, Guanine, Hypoxanthine, and Xanthine

Sample No.	Additions to 1 cc. blood			Incubation at 40-48°	Mg. uric acid per 100 cc. blood		
	Substrate		0.005 N NaOH		Found present	Formed from added substrate*	Lowered by presence of hypoxanthine or adenine†
	Purine dissolved in 0.01 N NaOH, 1 mg. per cc.	Amount					
		mg.	cc.	hrs.			
1a	None	0.0	0.0	0.0	8.75		
1b	"	0.0	1.0	3.0	10.90		
1c	Hypoxanthine	0.1	0.9	3.0	19.85	8.95	
1d	"	1.0	0.0	3.0	8.43	-2.47	2.47
1e	Xanthine	1.0	0.0	3.0	24.90	14.00	
1f	" and hypoxanthine	2.0‡	0.0	3.0	12.00	1.10	12.90
1g	Guanine	1.0	0.0	3.0	17.69	6.79	
1h	" and hypoxanthine	2.0‡	0.0	3.0	9.44	1.46	8.25
2a	None	0.0	0.0	0.0	4.90		
2b	"	0.0	1.0	2.5	5.56		
2c	Hypoxanthine	0.1	0.9	2.5	17.62	12.06	
2d	"	1.0	0.0	2.5	5.49	-0.07	0.07
2e	Xanthine	1.0	0.0	2.5	28.04	22.48	
2f	" and hypoxanthine	2.0‡	0.0	2.5	7.00	1.44	21.04
2g	Hypoxanthine	0.5	0.5	2.5	8.56	3.00	
2h	Xanthine	0.5	0.5	2.5	32.25	26.69	
2i	" and hypoxanthine	1.0‡	0.0	2.5	10.52	4.96	21.73
3a	None	0.0	0.0	0.0	4.58		
3b	"	0.0	0.0	2.5	6.61		
3c	"	0.0	1.0	2.5	7.00		
3d	Adenine	1.0	0.0	2.5	5.00	-2.00	2.00
3e	Guanine	1.0	0.0	2.5	22.50	15.50	
3f	Hypoxanthine	1.0	0.0	2.5	4.88	-2.12	2.12
3g	Xanthine	1.0	0.0	2.5	26.52	19.52	
3h	"	1.0	1.0	2.5	26.60	19.60	
3i	" and adenine	2.0‡	0.0	2.5	22.50	15.50	4.10

* The figures in this column were obtained by subtracting the amount of uric acid found present when the blood had incubated with alkali without the addition of purines from that found when the blood had incubated with purines.

TABLE V—*Concluded*

† The figures in this column were obtained by subtracting the amount of uric acid found present when adenine or hypoxanthine had been added for the incubation from that found under the same conditions when they had not been added.

‡ In these samples 1 mg. of each of the two purines was used except for Sample 2i in which only 0.5 mg. of each was used.

uric acid system (16), it is reasonable to assume that no xanthine would be converted to uric acid as long as considerable excess hypoxanthine is present and, in fact, a reduction of uric acid to xanthine would be expected under these conditions. Reindel and Schuler (14) observed that high concentrations of hypoxanthine reduced uric acid to xanthine in the presence of xanthine oxidase from rat kidney. In our experiments a decrease in uric acid was at times observed with 1 mg. of hypoxanthine (see Table V, Sample 1d). This shows that there has been no significant involvement of atmospheric oxygen. On the other hand, 0.1 mg. of hypoxanthine (0.74 micromole) is only slightly more than equivalent to the dissolved oxygen (0.4 micromole) and much less than that of the 8 micromoles of oxygen held as oxyhemoglobin. This small amount of hypoxanthine would consume only a small fraction of the total available oxygen; therefore, it would not only interfere with the oxidative conversion of the naturally existing xanthine to uric acid but itself would be oxidized to uric acid (see Table V, Samples 1c and 2e).

These points are illustrated still further in the data on mixed substrates. When the concentration of hypoxanthine is sufficiently high to use up the easily available oxygen, very little uric acid is formed either from hypoxanthine or xanthine, but when the concentration of hypoxanthine is lowered uric acid appears (compare Samples 2i and 2f of Table V).

The great increases in uric acid formation after addition of minute amounts of methylene blue (0.2 micromole) with 1 mg. of xanthine and 0.25 mg. of hypoxanthine (see Table III, Samples 2c and 2f) suggest that methylene blue increases the availability of the oxidizing action of the oxygen. There is sufficient oxygen in the blood (including the 8 micromoles held as oxyhemoglobin) to convert all the 1 mg. (7.4 micromoles) of hypoxanthine to uric acid; however, since 1 mg. of hypoxanthine causes only slight

TABLE VI

Uric Acid Content of Rat Blood When Incubated for 3 and 24 Hours with Various Quantities of Adenine in Presence and in Absence of Methylene Blue; and When Incubated with Hypoxanthine, Guanine, and Xanthine

Sample No.	Additions to 1 cc. blood				Incubation*	Mg. uric acid per 100 cc. blood	
	Substrate		0.005 N NaOH	0.001 M methyl-ene blue		Found present	Formed from added substrate†
	Purine dissolved in 0.01 N NaOH, 1 mg. per cc.	Amount					
		mg.	cc.	cc.	hrs.		
1a	None	0.00	0.00	0.0	0.0	8.75	
1b	"	0.00	1.00	0.0	3.0	10.90	
1c	Adenine	0.10	0.90	0.0	3.0	10.00	-0.90
1d	"	0.05	0.95	0.0	3.0	10.58	-0.42
1e	"	0.10	0.90	0.2	3.0	10.40	-0.50
1f	" ‡	0.10	0.90	0.0	3.0	10.34	-0.56
1g	None	0.00	1.00	0.0	24.0	13.63	
1h	Adenine	0.10	0.90	0.0	24.0	16.10	2.47
1i	"	0.25	0.75	0.0	24.0	16.10	2.47
1j	"	0.10	0.90	0.2	24.0	13.85	0.22
1k	"	0.25	0.75	0.2	24.0	14.00	0.37
1l	Guanine	1.00	0.00	0.0	3.0	17.69	6.79
1m	"	1.00	0.00	0.2	3.0	20.62	9.72
2a	None	0.00	0.00		0.0	5.00	
2b	"	0.00	0.00		3.0	6.38	
2c	"	0.00	1.00		3.0	7.46	
2d	Adenine	0.10	0.90		3.0	7.74	0.28
2e	"	0.10	0.00		3.0	7.45	1.07§
2f	None	0.00	1.00		24.0	8.43	
2g	Adenine	0.25	0.75		24.0	11.28	2.85
2h	"	0.10	0.90		24.0	10.81	2.38
2i	Hypoxanthine	1.00	0.00		24.0	8.54	0.11
2j	"	0.10	0.90		3.0	18.86	11.40
2k	"	0.25	0.75		3.0	14.04	6.58
2l	Guanine	0.25	0.75		3.0	17.97	10.51
2m	Xanthine	0.25	0.75		3.0	30.66	23.20

* The 3 hour incubation was at 40-48° and the 24 hour incubation at 38-40°.

† The figures in this column were obtained by subtracting the amount of uric acid found when purines had not been added from that found after the incubation with purines—the same time being allowed for incubation in both cases.

TABLE VI—*Concluded*

‡ This sample of adenine had previously been incubated for 24 hours at 38–40° to determine whether the incubation alone was responsible for the transformation into hypoxanthine.

§ This value is obtained by using as the blank value that formed without alkali (Sample 2b); the 0.1 cc. of alkali in which the adenine was dissolved was probably responsible for the increase observed.

increases in uric acid (see Table III, Sample 2d), it follows that methylene blue only partially increases the availability of oxygen held as oxyhemoglobin. It is also possible that the large amount of xanthine, formed by the first step of oxidation of hypoxanthine, as well as hypoxanthine itself, may inhibit in part the uric acid formation by a specific combination of the xanthine oxidase with the purines.

The effect of cyanide in inhibiting xanthine oxidase has been interpreted as a specific inactivation of the dehydrogenase (5). Some of our data, however, suggest that it inhibits, as does hypoxanthine, by acting as a reducing agent. It is true that with cyanide alone, the uric acid values are not markedly decreased, although with high concentrations of cyanide (40 micromoles) there are definite reductions (see Table III, Sample 1d). But in the presence of methylene blue (0.2 micromole), cyanide (20 micromoles) causes a marked decrease in uric acid concentration (this takes place only in the presence of xanthine oxidase). From this it seems that the enzyme catalyzes the reduction of uric acid by the cyanide. To act thus the cyanide must first remove all the dissolved oxygen. It is able to do this, since 20 micromoles of cyanide are in great excess of the 8 micromoles of oxygen (including that held as oxyhemoglobin) present in the blood. When the amount of cyanide is decreased to 10 micromoles (see Table III, Sample 1c) which is about equivalent to the total oxygen of the blood, it no longer causes a reduction of uric acid, although, as would be expected, it inhibits the formation of uric acid.

Effect of Adenine and Guanine on Formation of Uric Acid in Rat Blood

Adenine was listed by Coombs (17) as one of the purines which inhibits xanthine oxidase, and he explained the inhibition on the basis of adsorption of the purine on the surface of the enzyme.

In our experiments adenine, like hypoxanthine, in concentrations of 1 mg. per cc. of blood in 2.5 to 3 hours incubation inhibited the formation of uric acid in rat blood (see Table V, Samples 3d and 3f). This concentration of adenine also retarded the formation of uric acid from xanthine, but not so strikingly as did hypoxanthine (compare Samples 2f and 3i in Table V). On the other hand, in lower concentrations, 0.1 mg. per cc., at which hypoxanthine caused a great increase, adenine had very little effect — in some cases it caused a slight decrease (Table VI, Samples 1c and 1d compared with 1b) and in others (Samples 2d and 2c) a slight increase from that found without adenine. Methylene blue had no appreciable effect on these uric acid values (Sample 1e). In a longer time, 24 hours, adenine caused an increase in the uric acid value (Samples 1h and 1i, 2g and 2h) and this increase was almost completely prevented by the addition of methylene blue (Samples 1j and 1k).

Guanine, like xanthine, caused marked increases in uric acid formation, although the effects are not as great as those with xanthine (see Table VI, Samples 2l and 2m). Methylene blue increased the formation of uric acid from guanine (Samples 1l and 1m). The conversion of guanine into uric acid, like that of xanthine, was inhibited by hypoxanthine (Table V, Samples 1g and 1h).

The conversion of adenine and guanine into uric acid indicates the presence of adenase and guanase in rat blood. The increase in uric acid from adenine only after long incubation (24 hours) shows that adenase acts much more slowly than guanase or is present in much lower concentration.

Since xanthine oxidase has been shown to catalyze the direct oxidation of adenine without deamination (12), and if we assume that this oxidation which does not lead to uric acid is promoted by methylene blue, the other effects of adenine are readily understood. From this point of view, 1 mg. of adenine (like 1 mg. of hypoxanthine) would so deplete the available oxygen that the oxidative conversion of xanthine to uric acid would be markedly interfered with; while 0.1 mg. of adenine, being equivalent to much less than the total available oxygen, would have no significant effect. The speeding up of this insignificant effect of 0.1 mg. of adenine by methylene blue would not alter the magnitude and

thus would have no effect on the uric acid value. With large amounts of adenine (1 mg.) and methylene blue, one would expect less uric acid formation than without methylene blue because of the more complete removal of oxygen by the adenine. This was the observation in the one experiment of this type which was carried out—the uric acid value was 5.28 mg. per cent with 1 mg. of adenine and 4.81 mg. per cent with 1 mg. of adenine plus methylene blue. It remains to point out that the inhibition by methylene blue of the uric acid formation from adenine in 24 hours is explained by a methylene blue catalysis of the increased non-uric acid-forming oxidation of adenine. The adenine thus altered would otherwise be deaminized by the more slowly acting adenase, and thus be converted to uric acid.

In all the interpretations in this paper we have preferred a theory of relative concentrations of oxidizing and reducing agents and their oxidation-reduction potentials to a theory of specific inhibition or inactivation of enzyme by substrate or cyanide, because the former theory is more generally useful in predicting the phenomena presented here and more amenable to verification.

SUMMARY

1. The properties of xanthine oxidase of rat blood have been shown to be similar to those of xanthine oxidase of milk or other mammalian tissues. It catalyzes the oxidation of hypoxanthine as well as xanthine, and its action is promoted by methylene blue. It is inhibited by quinimine and by cyanide, and the cyanide inhibition is not prevented by methylene blue. There is a critical concentration of substrate at which the enzyme acts best; when the substrate is increased beyond this point, the action is retarded.

2. Methylene blue plus cyanide, and to a smaller extent cyanide alone, causes marked decreases in uric acid from that originally present, which indicates that the cyanide inhibition may in part be due to its action as a reducing agent.

3. In high concentrations both adenine and hypoxanthine markedly inhibit uric acid formation.

4. Adenine, in low concentrations, and in adequate time, increases uric acid; this indicates that adenase is present in rat blood. These increases from adenine are prevented by methylene blue.

5. An interpretation of the effects of cyanide, methylene blue,

hypoxanthine, and adenine is given chiefly in terms of a balance between the concentrations of oxidizing and reducing agents, rather than a specific adsorption on the surface of the enzyme.

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